

# Molecular Basis of the RhC<sup>w</sup> (Rh8) and RhC<sup>x</sup> (Rh9) Blood Group Specificities

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The Rh blood group antigens are encoded by two highly related genes, *RHD* and *RHCE*, and the sequence of the common alleles (*D*, *Ce*, *CE*, *ce*, and *cE*) of these genes has been previously elucidated. In this report, Rh transcripts and gene fragments have been amplified using polymerase chain reaction from the blood of donors with the C<sup>w</sup> and C<sup>x</sup> phenotypes. Sequence analysis indicated that the expression of the C<sup>w</sup> (Rh8) and C<sup>x</sup> (Rh9) antigens are associated with point mutations in the *RHCE* gene, which provides the definitive evidence that the C<sup>w</sup> and C<sup>x</sup> specificities are encoded by the same gene as the Cc and Ee antigens. As compared with the common (C<sup>w</sup>- and C<sup>x</sup>-) transcripts of the *RHCE* gene,

the C<sup>w</sup> and C<sup>x</sup> cDNAs exhibited A122G and G106A transitions that resulted in Gln41Arg and Ala36Thr amino acid substitutions in the C<sup>w</sup> and C<sup>x</sup> polypeptides, respectively. Therefore, although the C<sup>w</sup> and C<sup>x</sup> specificities behave serologically as if they were allelic, they cannot not be considered, *stricto sensu*, as the products of antithetical allelic forms of the *RHCE* gene. Based on the C<sup>w</sup>-/C<sup>w</sup>+ nucleotide polymorphism, a polymerase chain reaction assay useful for diagnosis purposes has been developed that detects the presence of the C<sup>w</sup> allele by the use of an allele-specific primer.

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**M**ANY OF THE 48 OR SO serologically defined RH (Rhesus) blood group system antigens are of low frequency. Thus, suitable families in which they segregate to allow the clarification of their genetic relationships are scarce. The low-frequency Rh antigens C<sup>w</sup> (Rh8) and C<sup>x</sup> (Rh9), with frequencies of about 2% and 0.1% in populations of general white extraction, respectively, are examples of such antigens.<sup>1,2</sup> Both are strongly associated and cosegregate in whites, with the common *DCE* gene complex producing, in addition, D, C, and e antigens. However, an allele for the C<sup>w</sup> antigen has also been reported to be rarely carried in gene complexes producing neither D and/or C,<sup>3-6</sup> whereas an allele for the C<sup>x</sup> antigen has been shown to be associated commonly with a novel gene complex (*dce*<sup>x</sup>) in the Somali population of East African origin.<sup>7</sup>

C<sup>w</sup> and especially C<sup>x</sup> are relatively common in the Finns, with a frequency of about 4% each,<sup>8</sup> thus greatly facilitating the ascertainment of families offering information as to the segregation of the antigens. A novel Rh antibody defining a high-frequency antigen MAR (Rh51) was recently shown in selected Finnish families to have an antithetical relationship to both anti-C<sup>w</sup> and anti-C<sup>x</sup>.<sup>8</sup> Defined by serologic methods, these three antigens behaved as they were determined by an allelic series of genes. Subjects with MAR-negative red blood cells (RBCs) were either homozygous for C<sup>w</sup> or C<sup>x</sup> or heterozygous C<sup>w</sup>/C<sup>x</sup>, whereas subjects with MAR-positive cells were homozygous or heterozygous for the common Rh gene complexes not expressing either C<sup>w</sup> or C<sup>x</sup> antigens.

Significant progress has been made recently regarding the structure and products of the RH blood group locus.<sup>9</sup> RhD-

positive chromosomes carry two closely linked genes, *RHD* and *RHCE*, that are inherited 'en bloc' from one generation to another, whereas RhD-negative chromosomes carry a single gene, *RHCE*.<sup>10</sup> This model predicted that the *RHD* gene should encode the RhD protein and that the *RHCE* gene should encode both C/c and E/e proteins, most likely after alternative splicing of a primary transcript.<sup>11</sup> Sequence analysis of transcripts from human reticulocytes and genomic DNA from individuals of different Rh phenotypes indicated that the D and non-D proteins exhibit 92% sequence homology<sup>12,13</sup> and also provided the genetic molecular basis for C/c E/e specificities.<sup>14</sup>

In this report, we have elucidated the molecular basis of the C<sup>w</sup> and C<sup>x</sup> antigens. This study gave new insights in the relationship between these low-frequency antigens and the Cc specificities and also provided a new tool for the detection of the C<sup>w</sup> allele.

## MATERIALS AND METHODS

**Materials.** Restriction enzymes were from Appligene (Strasbourg, France). The pBluescript II SK (+/-) vector was from Stratagene (La Jolla, CA). Radiolabeled <sup>32</sup>P-labeled nucleotides were from ICN Biomedicals (Amsterdam, The Netherlands). The first-strand cDNA kit containing the Moloney murine leukemic virus (M-MuLV) reverse transcriptase and the cycle sequencing kit were obtained from Pharmacia (Uppsala, Sweden). *Thermus aquaticus* polymerase (*Taq* polymerase) was from Perkin-Elmer-Cetus (Norwalk, CT).

**Blood samples.** Units of CPD buffy coats (leukocyte concentrate prepared by the closed Opti-system) from 2 homozygous C<sup>w</sup>+ patients (R.N. and I.K.), 1 homozygous C<sup>x</sup>+ patient (L.R.), and 1 heterozygous C<sup>w</sup>+C<sup>x</sup>+ patient (P.F.N.) with the DCCee phenotype<sup>8</sup> were provided by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). Blood samples from 4 C<sup>w</sup>+ donors with the Dccee phenotype (G.A., M.C.M., G.F., and J.A.) and 2 C<sup>w</sup>+ donors with the DCCee (A.M.) and DCCee (J.L.L.) phenotypes were collected on EDTA by the Centre National de Référence pour les Groupes Sanguins (CNRGS; Paris, France).

**Reverse transcription coupled with polymerase chain reaction amplification (RT-PCR).** Total RNAs were extracted by the acid-phenol-guanidinium method<sup>15</sup> from 5 mL of peripheral blood. Reverse transcription was performed at 37°C for 1 hour in a reaction mixture (15 µL) containing 1.8 mmol/L each dNTP, 4 mmol/L Tris pH 8.3, 68 mmol/L KCl, 15 mmol/L dithiothreitol (DTT), 9 mmol/L MgCl<sub>2</sub>, 0.08 mg/mL bovine serum albumin (BSA), 2 µg of random hexadeoxynucleotides, and 10 U of M-MuLV reverse transcriptase. One fourth of the cDNA products was subjected to PCR amplifica-

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Table 1. Primers Used in PCR Amplification

Primer	Sequence	Position (5', 3')*
P1	CATCTCCCCACCGAGC	Intron 1 (D, CE)
P2	CCAGCCACCATCCCAAT	Intron 2 (D, CE)
P3	GATGAGTCTAAGTACCCGCGG	-1, +21 (D, CE)
P4	ATGCCACGAGCCCTTTC	+139, +122 (C <sup>w</sup> )
P5	GAACACGTAGAAGTGCCTCAG	+525, +505 (CE)
P6	ACTACCACATGAACCTGAGGCAGT	+491, +514 (CE)
P7	GCTGTCTATGAGCGTTTCTC	+1349, +1331 (D, CE)

\* Position +1 is taken as the first nucleotide of the initiator (AUG). The specificity of primers for D, CE, and C<sup>w</sup> sequences is given in parentheses.

tion<sup>16</sup> in Taq buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 8.3, 0.001% gelatin [wt/vol]), 0.2 mmol/L of the four dNTPs, 50 pmol of each primer, and 2.5 U of Taq polymerase. Oligonucleotide sequences (Table 1) deduced from the human RhIXb cDNA clone<sup>11</sup> were as follows: set-1, P3-P5 (5' PCR fragments); and set-2, P6-P7 (3' PCR fragments). Thirty cycles of amplification were performed in a Perkin-Elmer-Cetus thermal cycler under the following conditions: denaturation at 92°C for 1 minute, primer annealing at 60°C for 1 minute, and extension at 72°C for 1.5 minutes. Amplified cDNA products were purified on agarose gels and then subcloned in pBluescript vectors.

**DNA sequencing.** Inserts from recombinant pBluescript vectors were sequenced on both strands by the dideoxy chain termination method<sup>17</sup> with a Pharmacia Cycle sequencing kit.

**PCR amplification with C<sup>w</sup> allele-specific primers (PCR-ASP).** Human DNA was extracted from 5 × 10<sup>6</sup> B lymphocytes (Epstein-Barr virus [EBV]<sup>+</sup>) or from 200 µL of total frozen blood with standard proteinase K (45 minutes at 56°C in Taq buffer containing 1% [vol/vol] Tween 20 and 100 µg/mL of proteinase K) and phenol-chloroform methods. For the PCR-ASP assays, one fifth of the DNA preparations was added in a 50 µL reaction volume (final concentrations of 100 mmol/L Tris-HCl, pH 8.3, 2.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 0.1 mg/mL gelatin, 0.2 mmol/L of each dNTP, 2.8% dimethylsulfoxide [DMSO], 0.5 mmol/L tetramethylammonium chloride [TMAC], 1 mmol/L of each primer, with 2.5 U of Taq polymerase). DMSO and TMAC were added to increase the specificity of the allele-specific primers and the hot start procedure was used (ie, Taq polymerase was added when the reaction mixture has been incubated for 10 minutes at 94°C). The following two sets of primers were used (Table 1): set-1, P3-P4 (C<sup>w</sup>-specific fragments); and set-2, P1-P2 (control fragments). The multiplex PCR reaction was performed under the following conditions: 32 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. One fifth of the PCR products was analyzed on a 2% agarose gel.

## RESULTS

**Phenotype status of the MAR-negative donors.** The 2 homozygous C<sup>w</sup>+ (R.N. and I.K.) and 1 homozygous C<sup>x</sup>+ blood donors (L.R.) were probands in the families described in Sistonen et al<sup>8</sup> (families 3, 8, and 1, respectively) and thus had their C<sup>w</sup>/C<sup>x</sup>/MAR phenotype confirmed also by typing the respective other family members. In each family the alleles for C<sup>w</sup> and C<sup>x</sup> cosegregated with the common gene complex DCE. For the MAR-negative C<sup>w</sup>+C<sup>x</sup>+ donor (P.F.N.), no family information was obtained. Other C<sup>w</sup>+ samples investigated were not typed for the MAR antigen.

**PCR amplification and sequence analysis of RhCE transcripts from C<sup>w</sup>+ and from C<sup>x</sup>+ donors.** Total RNAs ex-

tracted from peripheral blood of 1 homozygous C<sup>w</sup>+ donor (R.N.) and 1 homozygous C<sup>x</sup>+ donor (L.R.) with the DCCee phenotype and of 1 heterozygous C<sup>w</sup>+ individual (G.A.) with the Dccee phenotype were converted to cDNAs and then amplified by PCR. Amplifications were performed between two sets of primers designed to generate a 5' fragment (expected size, 527 bp) and a 3' fragment (expected size, 858 bp) specific for exons 1 to 4 and for exons 4 to 10 of the RHCE gene, respectively. Because the sequence of one of the two oligonucleotides that composed each set of primers was specific for the RHCE gene transcripts (P5 and P6; Table 1), only non-D cDNAs could be amplified. The PCR products were subcloned in pBluescript vectors and several recombinant clones derived from independent PCR experiments were analyzed to detect putative errors caused by the Taq polymerase activity.

The sequence of the Rh transcripts derived from the RHCE gene from the 2 C<sup>w</sup>+ donors and the C<sup>x</sup>+ donor was determined (Table 2). These sequences were compared with that of the previously described *ce*, *Ce*, *ce*, and *CE* alleles of the RHCE gene with C<sup>w</sup>- and C<sup>x</sup>- phenotypes.<sup>14</sup>

In all clones from the homozygous C<sup>w</sup>+ (DCCee) sample investigated, the same A → G transition was detected at nucleotide 122, which resulted in a Gln → Arg substitution at amino acid 41 of the RHCE-encoded polypeptides. Similarly, all clones from the homozygous C<sup>x</sup>+ (DCCee) sample contained a single base substitution located at nucleotide 106. The G → A transition resulted in an Ala → Thr substitution at amino acid 36. No other polymorphisms were detected in the remaining coding sequences as compared with the common *Ce* (C<sup>w</sup>-, C<sup>x</sup>-) allelic cDNAs. Because nucleotides 122 and 106 are both located in exon 1 of the RHCE gene,<sup>15</sup> the association of C<sup>w</sup> and C<sup>x</sup> with substitutions at these positions was confirmed after amplification of exon 1 on the genomic DNA from R.N., L.R., and other unrelated C<sup>w</sup>+ and/or C<sup>x</sup>+ donors (Table 2). Sequence analysis of these RHCE gene fragments indicated in all instances the presence of the same nucleotide polymorphisms in genomic DNAs as those observed in the cDNAs. As expected, the A122G and G106A polymorphisms were detected in all the clones from homozygous C<sup>w</sup>+ (R.N. and I.K.) and C<sup>x</sup>+ (L.R.) donors and in six clones derived from the heterozygous C<sup>w</sup>+C<sup>x</sup>+ donor (P.F.N.); some exhibited the A122G nucleotide substitution and the others contained the G106A polymorphism.

The C<sup>w</sup>-associated A122G substitution identified in the *Ce* allele (donors R.N., I.K., and P.F.N.) was also detected in the *ce* allele from the heterozygous C<sup>w</sup>+ (Dccee) donor (G.A.). In addition, as compared with the sequence of the common *ce*C<sup>w</sup>- allele previously published,<sup>14</sup> the *ce*C<sup>w</sup>+ allele exhibited another substitution, G48C. This mutation resulted in the presence of a cysteine at position 16, which was first associated with the expression of the Rh blood group antigen C.<sup>14</sup> Both the G48C and A122G polymorphisms in the *ce*C<sup>w</sup>+ allele were confirmed by analyzing the sequence of the PCR-amplified exon 1 from three unrelated C-C<sup>w</sup>+ donors (M.C.M., G.F., and J.A.; Table 2).

**PCR-based determination of the RhC<sup>w</sup> status.** The RhC<sup>w</sup> DNA typing was based on the A122G substitution identified

Table 2. Amino Acid Polymorphisms of the *RHCE*-Encoded Proteins Associated With the Expression of the  $C^w$  and  $C^x$  Specificities

Table 2. Amino Acid Polymorphisms of the <i>RHCE</i> -Encoded Proteins Associated with the Expression of the <i>RHCE</i> Gene							
		Status of the <i>RHCE</i> Gene Product at Amino Acid					
Sample	Transcripts	16 (nt 48)	36 (nt 106)	41 (nt 122)	60 (nt 178)	68 (nt 203)	103 (nt 307)
Controls							
C-c+E-e+C <sup>w</sup> -C <sup>x</sup> -	ce	W	A	Q	L	N	P
C+c-E-e+C <sup>w</sup> -C <sup>x</sup> -	Ce	C	A	Q	I	S	S
C-c+E-e-C <sup>w</sup> -C <sup>x</sup> -	cE	W	A	Q	L	N	P
Variants							
C+c-E-e+C <sup>w</sup> +C <sup>x</sup> - (R.N.)	Ce C <sup>w</sup>	C	A	R	I	S	S
C+c-E-e+C <sup>w</sup> +C <sup>x</sup> - (I.K.)	Ce C <sup>w</sup>	C	A	R	ND	ND	ND
C+c-E-e+C <sup>w</sup> -C <sup>x</sup> + (L.R.)	Ce C <sup>x</sup>	C	T	Q	I	S	S
C+c-E-e+C <sup>w</sup> +C <sup>x</sup> + (P.F.N.)	Ce C <sup>w</sup>	C	A	R	ND	ND	ND
	Ce C <sup>x</sup>	C	T	Q	ND	ND	ND
C-c+E-e+C <sup>w</sup> +C <sup>x</sup> - (G.A.)	ce C <sup>w</sup>	C	A	R	L	N	P
C-c+E-e+C <sup>w</sup> +C <sup>x</sup> - (M.C.M.)	ce C <sup>w</sup>	C	A	R	ND	ND	ND
C-c+E-e+C <sup>w</sup> +C <sup>x</sup> - (G.F.)	ce C <sup>w</sup>	C	A	R	ND	ND	ND
C-c+E-e+C <sup>w</sup> +C <sup>x</sup> - (J.A.)	ce C <sup>w</sup>	C	A	R	ND	ND	ND
		Exon1			Exon2		

Amino acids (one-letter code) associated with the expression of the  $C^w$  or  $C^x$  specificities are indicated in bold type. The C/c-associated polymorphisms previously described at amino acid positions 16, 60, 68, and 103 are indicated. The Cys16 present in the *RHCE*-encoded protein of four  $C-C^w+$  variants and usually associated with the expression of C is indicated in italics. Initials of the  $C^w+$  and/or  $C^x+$  donors are indicated in parentheses.

Abbreviations: ND, not determined; nt, nucleotide.

between the  $C^w-$  and  $C^w+$  alleles of the *RHCE* gene. A multiplex PCR-ASP assay was performed with two pairs of primers (P1-P2 and P3-P4; Fig 1A). Primer P3 and the  $C^w+$  allele-specific primer P4 (containing at its 3' end the polymorphic nucleotide  $C^{122}$ ) were designed to amplify a 140-bp sequence (exon 1) only from DNA carrying the  $C^w+$  allele. Because it has been previously shown that the *RHD* gene contains an adenine at position 122,<sup>13</sup> the 140-bp fragment cannot derive from amplification of an *RHD* sequence. Primers P1 and P2 encompassing sequences common to all the alleles of the *RHCE* and *RHD* were designed to amplify an internal PCR control fragment of 220 bp (exon 2). Results of the RhC<sup>w</sup> PCR-ASP performed with DNAs from six donors of different known Rh phenotypes are shown in Fig 1B. Primers P3/P4 were found to amplify the 140-bp fragment exclusively from DNA prepared from homozygous or heterozygous  $C^w+$  donors. In contrast, the 220-bp fragment amplified with primers P1/P2 was detected in all samples ( $C^w-$  and  $C^w+$ ). Unfortunately, a similar PCR-ASP strategy was unsuccessful for the RhC<sup>x</sup> DNA typing.

## DISCUSSION

We have shown in this report using mRNA and genomic DNA sequence analysis that the  $C^w+$  (7 unrelated samples) and  $C^x+$  (2 unrelated samples) phenotypes are associated with point mutations in exon 1 of the *RHCE* gene. These results provide the first evidence, at the molecular level, that the  $C^w$  and  $C^x$  specificities are encoded by the same gene that encodes the C/c (and E/e) antigens. As compared with the common Rh proteins ( $C^w-C^x-$ ), the  $C^w+$  and  $C^x+$  polypeptides exhibit a Gln41Arg and an Ala36Thr substitutions, respectively. These residues are both located in the first extracellular loop of the Rh polypeptides (Fig 2) pre-

dicted from hydropathy plot analysis and immunochemical studies.<sup>19,20</sup> These residues should therefore be available for the binding of anti- $C^w$  and anti- $C^x$  antibodies on intact RBCs. Following this observation, synthetic peptides representing residues 34 to 46 of the  $C^w-C^x-$ ,  $C^w+$ , and  $C^x+$  Rh polypeptides were used in hemagglutination inhibition experiments with anti- $C^w$  and anti- $C^x$  antibodies against  $C^w+$  and  $C^x+$  erythrocytes (data not shown). No inhibition was observed, indicating that linear peptides are unable to mimic the  $C^w$  and  $C^x$  antigens, most likely because these specificities are carried by conformation-dependent structures. This finding is consistent with recent studies of a rare variant carrying the  $DC^w-$  gene complex<sup>21</sup> in which DNA exchange between the *RHD* and the *RHCE* genes is responsible for the lack of sequences specific for exons 3 to 9 of the *RHCE* gene. The *RHCE-D-CE* hybrid gene that results from this gene conversion event encodes for an Rh polypeptide carrying the Gln41Arg substitution.<sup>21</sup> However, the  $DC^w-$  RBCs studied exhibited a reduced expression of the  $C^w$  antigen as compared with  $C^w+$  erythrocytes of common Rh phenotypes. These findings suggest that different regions of the *RHCE*-encoded proteins, in addition to the first extracellular loop, might be involved in the full expression of the  $C^w$  antigen.

For many years after their discovery, the  $C^w$  and  $C^x$  specificities were regarded as the products of alleles  $C^w$  and  $C^x$  at the Cc locus or sublocus.<sup>22</sup> Recently, population and family studies on Finnish blood donors indicated that  $C^w$  and  $C^x$  belong to the same allelic series with a novel high-incidence Rh antigen MAR (Rh51).<sup>8</sup> The anti-MAR antibodies, which were found to be present in the serum of a rare heterozygous  $C^w/C^x$  donor (typed as MAR negative), were shown to be antithetical to anti- $C^w$  and anti- $C^x$  antibodies. Because no

antithetical relationship between those antibodies and antibodies to the C/c or E/e antigens could be established, it was suggested that C<sup>w</sup>/C<sup>x</sup>/MAR behave genetically as a separate allelic subsystem. However, the sequence analysis reported here indicated that, in contrast to the C/c (Ser-103Pro) or E/e (Pro226Ala) specificities,<sup>14</sup> the C<sup>w</sup> and C<sup>x</sup> specificities should not be considered, *stricto sensu*, as the products of antithetical allelic forms of the *RHCE* gene, because the substitutions associated with their expression (A122G and G106A, respectively) are not located at the same nucleotide position. Therefore, it cannot be excluded that a very infrequent crossing over might occur between the two polymorphic nucleotides. An *RHCE* gene resulting from this putative recombination has not yet been identified. It is not known whether such a gene should encode for both the C<sup>w</sup> and C<sup>x</sup> specificities or whether the presence of the Gln41Arg and Ala36Thr substitutions on the same Rh polypeptide would result in the loss of C<sup>w</sup> and C<sup>x</sup> and/or in the expression of a still-uncharacterized Rh antigen. Because the MAR antigen is expressed only at the surface of erythrocytes

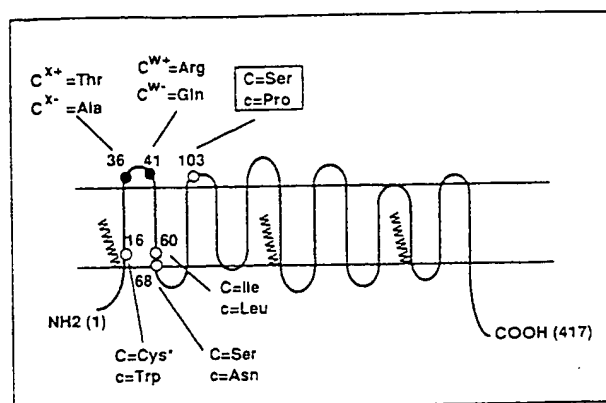
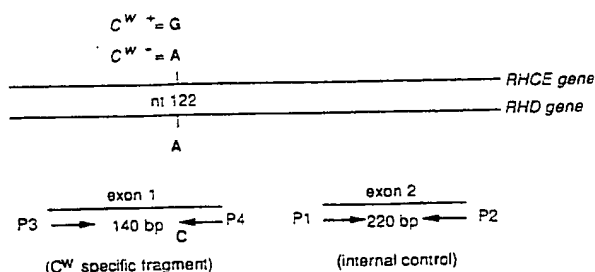


Fig 2. Localization of the C<sup>w</sup> and C<sup>x</sup>-associated polymorphisms on the Rh protein. The predicted membrane topology of the *RHCE* gene encoded protein is represented. (O) Amino acids generally associated with the C/c polymorphisms.<sup>16</sup> Amino acids critical for the expression of C or c are boxed. (●) Amino acids associated with the C<sup>w</sup>-/C<sup>w</sup>+ and C<sup>x</sup>-/C<sup>x</sup>+ and located in the first extracellular loop. Putative palmitate fatty acid chains linked to cysteine residues of Cys-Leu-Pro motifs are indicated. (\*) The Cys residue at position 16 associated with the RhC protein is also found in Rhc from rare C-C<sup>w</sup>+ variants as well as in some Rh-negative donors (dccc).

A



B

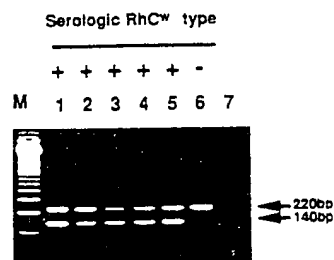


Fig 1. RhC<sup>w</sup> DNA typing. (A) Strategy of the PCR-ASP. The 220-bp product amplified from all the *RH* genes between primers P1 and P2 acts as an internal control of the PCR reactions. The 140-bp product can be amplified only from the C<sup>w</sup>+ allele of the *RHCE* gene between primer P3 and the allele-specific primer P4. The nucleotides found at the polymorphic position 122 of the C<sup>w</sup>+ and C<sup>w</sup>- *RHCE* alleles and of the *RHD* gene are indicated. (B) The DNA from C<sup>w</sup>-typed donors was used as templates in the PCR-ASP assay. Lane 1, homozygous C<sup>w</sup>+ (DCCee); lane 2, C<sup>w</sup>+C<sup>x</sup>+ (DCCee); lanes 3 and 4, heterozygous C<sup>w</sup>+ (DCCee and DCCee); lane 5, C-C<sup>w</sup>+ (DCCee); lane 6, C<sup>w</sup>- (dccc); lane 7, negative control of the PCR reaction. PCR products were separated on a 2% agarose gel. Migration positions of the control 220-bp fragment (PCR between P1/P2) and of the 140-bp C<sup>w</sup>-specific fragment (PCR between P3/P4) are indicated. M, 100-bp ladder (markers).

heterozygous or homozygous for the common *RHCE* alleles not expressing either C<sup>w</sup> or C<sup>x</sup>, our results also indicate that this epitope is most likely associated with the presence of Ala36 and Gln41 on the common *RHCE*-encoded polypeptides. However, a synthetic peptide encompassing residues 34 to 46 that carries Ala36 and Gln41 did not inhibit anti-MAR sera (data not shown), again indicating that the MAR epitope is conformation dependent.

The C<sup>w</sup> and C<sup>x</sup> specificities were originally regarded as products of a gene encoding for both the C<sup>w</sup> and the C antigens or the C<sup>x</sup> and C antigens.<sup>22</sup> It is now known that both can also be produced by genes that encode for the C<sup>w</sup> and c or C<sup>x</sup> and c specificities.<sup>4,7,23</sup> Nucleotide sequence analysis of a rare C-C<sup>w</sup>+ donor was very useful in understanding the molecular basis of this phenotype. Indeed, the C<sup>w</sup>+ molecule of these variants is composed of a C-terminal region encoded by exons 2 to 10 of the *ce* allele, whereas the N-terminal domain is encoded by exon 1 of an allele that exhibited the two polymorphisms (at positions 16 and 41) associated with the expression of the C and C<sup>w</sup> antigens (Table 2). These results strongly suggested that the molecular mechanism that would account for the C-C<sup>w</sup>+ phenotype must be an interallelic recombination involving sequences located within intron 1 of the *CeC*<sup>w</sup>+ and *ceC*<sup>w</sup>- allelic form of the *RHCE* gene. A similar recombination event has been proposed to explain the presence (in 6 of 102 randomly selected C-negative donors) of a Cys16 residue as well as of a *Msp*I restriction site in intron 1 of the *RHCE* gene,<sup>24</sup> which are generally associated with the expression of C. That amino acid change at position 16 is not strictly correlated with the C/c polymorphism was recently supported by nucleotide sequencing of transcripts isolated from the blood of several monkey species serotyped as c<sup>-</sup> or c<sup>+</sup>.<sup>25</sup> This analysis indicated that, among the four amino acid substitu-

tions (positions 16, 60, 68, and 103) first associated with the C/c polymorphism in humans.<sup>14</sup> only polymorphism at the exofacial position 103, located in the postulated second extracellular loop, was strictly correlated with the expression or the nonexpression of the Rhc antigen in nonhuman primates. However, it is noteworthy that in all C<sup>w</sup>+ samples investigated, whatever their C or c phenotypes, a cysteine residue was conserved at position 16 that is adjacent to a potential fatty acylation site, the Cys-Leu-Pro motif,<sup>26</sup> centered on the nonpolymorphic cysteine at position 12 (Fig 2). Rh polypeptides are major palmitoylated components of human RBC membranes and the presence of an extra lipid molecule might affect antigenic expression.<sup>27</sup> Whether the presence or the absence of a cysteine at position 16 may interfere with the potential palmitate anchor site at position 12 and therefore modulate expression of antigens that are dependent, as C<sup>w</sup>, on amino-acid polymorphisms within the first exofacial loop of the Rh polypeptide requires further investigation.

Since its discovery, the C<sup>w</sup> specificity was shown to be responsible for alloimmunizations. C<sup>w</sup>- subjects (whatever their C or c phenotypes) can form anti-C<sup>w</sup> antibodies as a result of transfusion with C<sup>w</sup>+ blood or pregnancies with C<sup>w</sup>+ fetuses. In few cases, anti-C<sup>w</sup> has caused hemolytic transfusion reactions and hemolytic disease of the newborn.<sup>28</sup> In cases of hemolytic disease of the newborn, an early diagnosis of fetal anemia is of prime importance for the success of in utero transfusions or exchange transfusions.<sup>29</sup> PCR experiments have been already developed to determine the fetal RhD, Rhc, and RhE types in DNA from amniotic and trophoblastic cells.<sup>30,31</sup> We have described the development of a PCR assay that detects in leukocyte genomic DNA the presence of the C<sup>w</sup>+ allele by the use of an allele-specific primer. It is assumed that, when performed with amniotic or trophoblastic cells, this PCR-based determination of the C<sup>w</sup> status of fetuses will prove to be useful for a proper management of pregnancies in highly C<sup>w</sup> immunized mothers.

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#### REFERENCES

1. Callender ST, Race RR: A serological and genetical study of multiple antibodies formed in response to blood transfusion by a patient with lupus erythematosus diffusum. *Ann Eugen* 13:102, 1946
2. Stratton F, Renton PH: Haemolytic disease of the newborn caused by a new Rh antibody, anti-C<sup>x</sup>. *Br Med J* 1:962, 1954
3. Gunson HH, Donohue WL: Multiple examples of the blood genotype C<sup>w</sup>D-/C<sup>w</sup>D- in a Canadian family. *Vox Sang* 2:320, 1957
4. Habibi B, André J, Fouillade MT, Lopez M, Salmon C: An unusual Rh phenotype indicating heterogeneity of the C<sup>w</sup> antigen. *Vox Sang* 31:103, 1976
5. Sachs HW, Reuter W, Tippet P, Gavin J: An Rh gene complex producing both C<sup>w</sup> and c antigen. *Vox Sang* 35:272, 1978
6. Giannetti M, Stadler E, Rittner C, Lomas C, Tippet P: A rare haplotype producing Cw and c, and D and e in a German family. *Vox Sang* 44:319, 1983
7. Sistonen P, Aden Abdulle O, Sahid M: Evidence for a 'new' Rh gene complex producing the rare C<sup>x</sup> (Rh9) antigen in the Somali population of East Africa. *Transfusion* 27:66, 1987
8. Sistonen P, Sareneva H, Pirkola A, Eklund J: MAR, a novel high-incidence Rh antigen revealing the existence of an allelic sub-system including C<sup>w</sup> (Rh8) and C<sup>x</sup> (Rh9) with exceptional distribution in the Finnish population. *Vox Sang* 66:287, 1994
9. Cartron JP: Defining the Rh blood group antigens: biochemistry and molecular genetics. *Blood Rev* 8:199, 1994
10. Colin Y, Chérif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP: Genetic basis of the RhD-positive and RhD-negative blood group polymorphism. *Blood* 78:2747, 1991
11. Le Van Kim C, Chérif-Zahar B, Raynal V, Lopez M, Cartron JP, Colin Y: Multiple Rh mRNAs isoforms are produced by alternative splicing and poly(A) site choice. *Blood* 80:1074, 1992
12. Le Van Kim C, Mouro I, Chérif-Zahar B, Raynal V, Chermier C, Cartron JP, Colin Y: Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proc Natl Acad Sci USA* 89:10925, 1992
13. Arce MA, Scott Thompson E, Wagner S, Coyne KE, Ferdman BA, Lublin DM: Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood* 82:651, 1993
14. Mouro I, Colin Y, Chérif-Zahar B, Cartron JP, Le Van Kim C: Molecular genetic basis of the human Rhesus blood group system. *Nat Genet* 5:62, 1993
15. Chomczynski P, Sacchi N: Single-step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156, 1987
16. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487, 1988
17. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463, 1977
18. Chérif-Zahar B, Le Van Kim C, Rouillac C, Raynal V, Cartron JP, Colin Y: Organization of the gene encoding the human blood group RhCcEe antigens and characterization of the promoter region. *Genomics* 19:68, 1994
19. Avent ND, Butcher SK, Liu W, Mawby WJ, Mallison G, Parsons SF, Anstee DJ, Tanner MJA: Localization of the C-termini of the Rh (Rhesus) polypeptides to the cytoplasmic face of the human erythrocyte membrane. *J Biol Chem* 267:15134, 1992
20. Hermand P, Mouro I, Huet M, Bloy C, Suyama K, Goldstein J, Cartron JP, Bailly P: Immunochemical characterization of Rhesus proteins with antibodies raised against synthetic peptides. *Blood* 82:669, 1993
21. Chérif-Zahar B, Raynal V, D'Ambrosio AM, Cartron JP, Colin Y: Molecular analysis of the structure and expression of the RH locus in individuals carrying D--, Dc-, DC<sup>w</sup>- gene complexes. *Blood* 84:4354, 1994
22. Issitt PD: *Applied Blood Group Serology* (ed 3). Miami, FL, Montgomery Scientific, 1985
23. Leonard GL, Ellis SS, Reid ME, Sanchez PD, Tippet P: An unusual Rh immunization. *Vox Sang* 31:275, 1976
24. Wolter LC, Hyland CA, Saul A: Refining the DNA polymorphisms that associate with the Rhesus c phenotype. *Blood* 84:985, 1994
25. Salvignol I, Calvas P, Socha WW, Colin Y, Le Van Kim C, Bailly P, Ruffié J, Cartron JP, Blancher A: Structural analysis of the RH blood group gene products in nonhuman primates. *Immunogenetics* 41:271, 1995
26. Hartel-Schenk S, Agre P: Mammalian red cell membrane Rh polypeptides are selectively palmitoylated subunits of a macromolecular complex. *J Biol Chem* 267:5569, 1992
27. de Vetten MP, Agre PJ: The Rh polypeptide is a major fatty

acid acylated erythrocyte membrane protein. *J Biol Chem* 263:18193, 1988

28. Lawler SD, van Loghem JJ: The Rhesus antigen C<sup>w</sup> causing haemolytic disease of the newborn. *Lancet* 2:545, 1947

29. Poissonnier MH, Brossard Y, Demeideros N, Vassileva J, Parnet F, Larsen M, Gosset M, Chavinié J, Huchet J: Two hundred intra uterine exchange transfusion in severe blood incompatibilities. *Am J Obstet Gynecol* 161:709, 1989

30. Bennett PR, Le Van Kim C, Colin Y, Warwick R, Cherif-Zahar B, Fisk NM, Cartron JP: Prenatal determination of fetal RhD type by DNA amplification. *N Engl J Med* 329:607, 1993

31. Le Van Kim C, Mouro I, Brossard Y, Chavinié J, Cartron JP, Colin Y: PCR-based determination of Rhc and RhE status of fetuses at risk of Rhc and RhE haemolytic disease. *Br J Haematol* 88:193, 1994